Endosomal pH-destabilized PGA-PCL block copolymer micelle

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Poly(L-glutamic acid)-poly(ε-caprolactone) diblock copolymers were prepared using ring opening polymerization. The diblock copolymers were characterized by 1H-NMR, gel permeation chromatography and FT-IR. Endosomal pH-destabilized nanoparticles were prepared from dialysis method and their micellar structure was confirmed by dynamic light scattering and transmission electron microscopy. In this study, the method of fabrication and pH-dependent structural changes of the diblock copolymer were investigated. pH-Dependent changeable nanoparticles may be helpful for intracellular delivery in drug delivery systems.

Key words: Poly(L-glutamic acid), poly(ε-caprolactone), micelle

Introduction

Nanoparticles are a promising candidate in field of drug delivery systems because they enhance the cellular uptake and reduce the side effects of drugs. They also make it possible to deliver a drug to specific cells.1-3 To increase the therapeutic efficacy of a drug, two main strategies can be considered. Firstly, receptor-mediated nanoparticles were prepared to increase the intracellular trafficking.4,5 Secondly, a therapeutic drug was released from endosome or lysosome to the cytoplasm or nucleus after being taken up by the cells. Endosome and lysosome are acidified by proton-translocating ATPases to below pH 5. At this stage, nanoparticles should release their contents prior. To accomplish this, several studies have been conducted using pH-sensitive liposomes, pH-sensitive micelles, etc.5-8

In many cases, poly(ethylene glycol) (PEG) has been used to achieve a long circulation time in the body, but it is difficult to release in a specific environment, such as in endosome or lysosome. Our objective is to make nanoparticles that release a drug at the endosomal pH. To achieve this purpose, the pH-sensitive nanoparticles, formed by poly(L-glutamic acid)-poly(ε-caprolactone) (PGA-PCL) diblock, were developed. PGA, one of the poly(α-amino acids), has a suitable ionization character to be applied to the release of a therapeutic drug at endosomal pH. The pKa value of PGA is approximately 5 and its solubility is changed from soluble to insoluble below pH 5, because it is transformed from random coil to α-helix.9,10 Therefore, its pH-dependent transition is suitable for transformation at endosomal pH below 5. When PGA is conjugated to PCL, a representative hydrophobic polymer, the resulting diblock copolymer can form nanoparticles, because PGA stabilizes the PCL surface. However, the nanoparticles are aggregated by hydrophobic interaction and structural deformation when PGA is deionized below pH 5, due to its change from random coil to α-helix structure, and therefore can release a loaded drug from the PCL core.

Block copolymers composed of PGA or poly(γ-benzyl glutamate) (PBLG) and PCL have already been synthesized but, to the best of our knowledge, their fabrication and utilization have not previously been reported. Herein, we studied the method of fabrication and pH-dependent structural changes of such block copolymer.

Materials and method

ε-Caprolactone (CL) was purchased from Aldrich. It was dried over CaH2 for 1 day and distilled at reduced pressure prior to use. Chloroform (CHCl3), methylene chloride (MC), tetrahydrofuran (THF), stannous octoate (Sn(Oct)2), L-glutamic acid, diethyl ether, sulfuric acid, 4-nitrophenethylalcohol (4-NPA), phenylmethanol, triphosgene, HBr (33% in acetic acid) and methanol were purchased from Aldrich. γ-benzyl-glutamate and γ-benzyl L-glutamate N-carboxyanhydride (Bn-Glu-NCA) were synthesized according to the reported procedures.13,14

PGA-PCL diblock copolymers were synthesized by a modified version of the method described by Rong et al.11 As
shown in Scheme 1, 4-NPA (2 mol% of CL, 0.60 g) and Sn(Oct)$_2$ (0.01 g) were added to a two-neck round-bottom flask, and then CL (20.6 g) was added under a nitrogen atmosphere. The reactant mixture was dried for 1 h under a vacuum at 60°C, and then the temperature was raised slowly to 135°C and the reaction carried out for 18 h. The synthesized PCL-NO$_2$ was dissolved in MC, and then the polymer solution was precipitated in excess methanol. The precipitated products were dried under a vacuum for 2 days.

10 g of PCL-NO$_2$, 40 mL of dry THF, and 1 g of palladium on charcoal (10% Pd/C) were transferred into a two-neck round-bottom flask which was then bubbled with hydrogen gas for 4 h. The resulting solution was filtered, precipitated in diethyl ether and dried in a vacuum at room temperature for 2 days and the purified amine terminated PCL was obtained.

Bn-Glu-NCA (10, 20, 50 mol% of PCL-NH$_2$) and PCL-NH$_2$ (1 g) were dissolved in 5 mL of CHCl$_3$, separately. The Bn-Glu-NCA solution was added to the PCL-NH$_2$ solution and the reaction mixture was continuously stirred at room temperature for 24 h. Then, the reaction solution was precipitated in excess methanol. After filtering, the solid was dried under a vacuum at room temperature for 2 days and PBLG-PCL block copolymer was obtained. Further, the benzyl group was deprotected by the method using acetic acid/HBr and PGA-PCL block copolymer was obtained.\(^{15}\)

To prepare nanoparticles, 10 wt% polymer solution in DMF was poured into 0.1 N NaOH/phosphate buffered saline (PBS) solution under vigorous stirring. The final polymer concentration was 1 wt%. Then, the solution was dialyzed against pH 7.4 PBS solution using a dialysis membrane (molecular weight cutoff 12,000 – 14,000) at 10°C overnight.

\(^{1}H\)-NMR spectra were recorded on a Varian-Unity Inova 500NB operated at 500 MHz and were used to determine the molecular structure and composition of the block copolymers. CDCl$_3$ was used as a solvent, including 0.03 vol. % tetramethylsilane (TMS). FT-IR spectra were recorded on Nicolet 380 FT-IR instrument. The molecular weights of the synthesized polymers were measured by gel permeation chromatography (GPC). THF was used as an eluent at a flow rate of 1 mL/min. KF-803L and KF-802.5 (Shodex) columns were used in series. The data were analyzed by means of an RI detector (RI-101, Shodex). Poly(ethylene glycol) standards (Waters) were used to determine the molecular weight. DLS (Dynamic Light Scattering, Malvern Instrument Ltd. Series 4700), with a helium laser at 633 nm and a digital correlator, was used to determine the size of the nanoparticles. For the TEM (transmission electron microscope) experiments, the sample solution was dropped onto a carbon coated copper grid. Excess solution was wicked away with filter paper and the sample was allowed to dry in air. When a negative staining technique was used to prepare the TEM samples, 1% uranyl acetate solution was dropped onto the sample. The sample was air dried and studied using a JEM-3010 electron microscope with a Gatan digital camera. To evaluate the pH-sensitivity, the turbidity of the polymer solution was measured by means of a UV-VIS spectrometer at various pH values, and titrated using 1 N NaOH aqueous solution. The turbidity change of the polymer solutions was determined from the light transmittance at $\lambda = 440$ nm.

**Results and discussion**

The polymerization of PCL-NO$_2$, initiated by 4-NPA, and the reduction of PCL-NO$_2$ to PCL-NH$_2$ were confirmed by
H-NMR. As shown in Figure 1, the phenyl protons were shifted from 8.2 and 7.4 to 7.0 and 6.6, respectively. The molecular weight of PCL-NH$_2$ was calculated by comparing the integrated value of the peaks for the -CH$_2$- proton of 4-phenetylamine ("i proton" in Figure 1-(b)) with that for the -CH$_2$- proton of PCL ("f proton"), as well as by GPC. The block copolymerization, with Bn-Glu-NCA, was confirmed by $^1$H-NMR and the molecular weight was determined from the peaks for the -CH$_2$- proton of PBLG ("k proton" in Figure 2) and -CH$_2$- proton of PCL ("a proton"). In the case of PCL-PBLG, GPC was not used due to the aggregation of PCL-PBLG block copolymer in organic solvents, as mentioned in a previous report.$^{16}$ As shown in Table 1, PBLG$_{100}$-PCL$_{50}$ and PBLG$_{20}$-PCL$_{50}$ diblock copolymers were well-synthesized, but the synthesis of PBLG$_{100}$-PCL$_{50}$ diblock copolymers failed. As previously reported,$^{16}$ the high molecular weight product was not acquired because the polymerization from NCA monomer has no living character. As shown in Figure 3, the deprotection of the benzyl group was confirmed by FT-IR spectroscopy and the absorbance peak of the phenyl group at 750 cm$^{-1}$ was disappeared.

The nanoparticles were fabricated using the crew-cut aggregate method of Eisenberg.$^{16}$ When the polymer solution in DMF was poured into 0.1 N NaOH PBS solution, the solution became slightly turbid, but no aggregates or sedimentation appeared.

Figure 1. $^1$H-NMR spectra of (a) PCL-NO$_2$ and (b) PCL-NH$_2$.

Table 1. Molecular weight of synthesized polymers

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<th>Nomenclature</th>
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<td>$M_n$ of PCL</td>
<td>$M_n$ of PBLG-PCL</td>
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Figure 2. $^1$H-NMR spectrum of PGA-PCL.
was observed. After dialysis in pH 7.4 PBS buffer, the solution became bluish and transparent because the polymer could form nanoparticles in the aqueous media. In the particle preparation procedure, only PGA$_{10}$-PCL$_{50}$ diblock copolymer was investigated. Figure 4 shows the TEM image and DLS results of PGA$_{10}$-PCL$_{50}$ diblock copolymer in buffer solution of pH 7.4. DLS shows a unimodal distribution of nanoparticles with a size of 60 nm, whereas the TEM image shows that the size was below 100 nm but with a relatively broader distribution. It seems that nanoparticles were contracted by the loss of water during the sampling and drying procedure of TEM experiment.

Figure 5 shows the transmittance of PGA$_{10}$-PCL$_{50}$ diblock copolymer nanoparticle at various pH. The transmittance of the polymer solution was measured using a UV-VIS spectrometer to determine the destabilization behavior. Figure 5 shows that the solution was clear above pH 6 but aggregated below pH 5. Only sedimentation was observed below pH 5. It is hypothesized that the anionic surface of the nanoparticles, resulting from the ionization of PGA, stabilizes the nanoparticles above pH 5 and that their destabilization results from the deionization of PGA and the transformation of the random coil to an α-helix below pH 5 due to the increased hydrophobic interaction between the nanoparticles and hydrogen bonding between the PGA molecules. In previous papers, it is reported that PGA transforms from a random coil to an α-helix between pH 6 and 4. It is considered that our result corresponds to the transformation character of PGA and this kind of character could be used to facilitate the release of drugs from endosome or lysosome having low pH.

**Conclusions**

It was concluded that the nanoparticles have the properties of pH-dependent destabilization from this study. The carboxyl group of PGA block can be easily modified by the introduction of poly(ethylene glycol) for a long circulation in the body. In addition, in order to increase the cellular uptake and achieve specific targeting, targeting molecules could be easily introduced into the block copolymer. Therefore, PGA-PCL diblock copolymers will be helpful for development of more efficient drug delivery systems.
Acknowledgements

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References